Efficacy of a Proapoptotic Peptide towards Cancer Cells

CLARA E. JÄKEL[1], KAROLINE MESCHENMOSER[2], YOUNG KIM[2],
HANS WEIHER[1] and INGO G.H. SCHMIDT-WOLF[2]

[1]*University of Applied Sciences Bonn-Rhein-Sieg, Immunology and Cell Biology, Rheinbach, Germany;*
[2]*Department of Internal Medicine III, Center for Integrated Oncology (CIO),*
University Hospital Bonn, Bonn, Germany

Abstract. *Background: Conventional cancer therapies are associated with severe side-effects and the development of drug resistance. Therefore, new strategies to specifically target tumor cells leaving healthy tissue unaffected are of great interest. Materials and Methods: On this respect, we tested the antimicrobial peptide (KLAKLAK)$_2$. Results: This peptide exhibits cytotoxicity against human breast cancer and other tumor cells, while healthy cells remain unaffected. Moreover, treatment with this cationic amphipathic peptide results in slower tumor growth and longer overall survival in vivo. Conclusion: Our data suggest a potential use of (KLAKLAK)$_2$ peptide for patients with breast and other types of cancer.*

Cancer is one of the leading causes of death worldwide; in the US, cancer accounted for nearly 25% of all deaths in 2009/2010 (1). Regarding all cancer types, breast cancer is the second most common cause of death after lung cancer among women in the US (2).

Conventional tumor therapies are relatively unspecific and affect not only tumor but also healthy cells. Thus, these therapies are accompanied by severe, dose-limiting side–effects. Therapies specifically targeting tumor cells leaving healthy cells unaffected could overcome these problems.

In recent years, the use of antimicrobial peptides (AMP) in cancer therapy has gained great interest. These peptides play an important role in host defense as they are effective against a broad spectrum of microbes, such as gram-positive and gram-negative bacteria, mycobacteria, fungi, parasites, enveloped viruses and also transformed cells (3, 4). Different AMPs can be found in virtually all higher eukaryotes and can be divided into two major groups: The first one comprises AMPs which are toxic to bacteria and tumor cells but not to healthy mammalian cells. The second group includes AMPs toxic to bacteria and mammalian tumor and non-tumor cells. The advantage of AMPs in tumor therapy is their broad range of activity, exhibiting toxicity against primary tumors as well as prevention of metastases, while being nontoxic to vital organs (3).

Cationic amphipathic AMPs preferentially bind to negatively charged membranes, such as bacterial outer membranes, by electrostatic and hydrophobic interactions, resulting in distortion of the membrane. In contrast, as eukaryotic plasma membranes are generally neutral, these peptides exhibit low cytotoxicity towards mammalian cells (5, 6). Furthermore, the presence of cholesterol reduces binding of the AMPs to mammalian plasma membranes (7-9) (Figure 1).

One cationic AMP which is often used for approaches in cancer therapy is the amphipathic α-helical peptide (KLAKLAK)$_2$. This peptide was shown to be able to distort the mitochondrial membrane (10). Mitochondria are key regulators in apoptosis and have thus become an attractive target for cancer therapy. Coupled to different targeting domains, this peptide was shown to specifically induce apoptosis in cancer cells (10-13).

In this study, we show that the (KLAKLAK)$_2$ peptide alone specifically reduces the viability of breast cancer and also other tumor cells, while healthy controls remain unaffected. Moreover, we show that this peptide retards tumor growth and prolongs survival *in vivo*.

Materials and Methods

Cell culture. All cells were incubated at 37°C in 5% CO_2 and 80% humidity. MDA-MB435S, T24 and DU145 cells were purchased from Cell Lines Service, Eppelheim, Germany. MDA-MB435S cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine (Invitrogen, Darmstadt, Germany), 1% penicillin/streptomycin (PAA Laboratories, Pasching, Germany), 5% heat inactivated fetal calf serum (FCS; Invitrogen) and 0.2%

Correspondence to: Professor Dr. Ingo G.H. Schmidt-Wolf, Department of Internal Medicine III, Center for Integrated Oncology (CIO), University Hospital Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany. Tel: +49 22828715507, Fax: +49 22828715849, e-mail: Ingo.Schmidt-Wolf@ukb.uni-bonn.de

Key Words: Antimicrobial peptides, (KLAKLAK)$_2$, targeted tumor therapy, MDA-MB435S, T24, DU145, Tet21N, Wac2 cells, breast cancer xenografts.
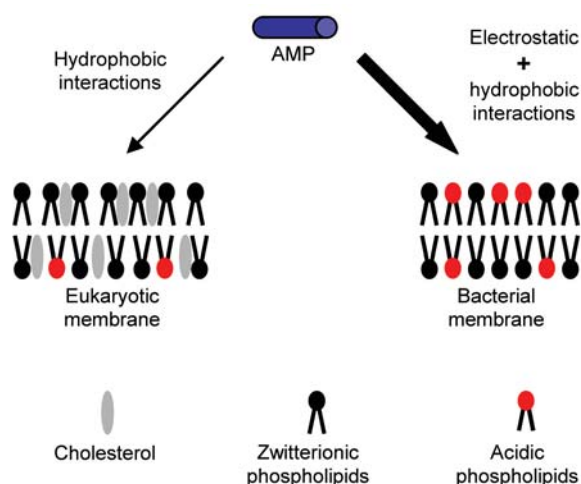
Figure 1. *Molecular basis of selective interaction of antimicrobial peptides (AMPs) with negatively charged membranes. Cationic AMPs strongly interact with acidic phospholipids abundant on bacterial membranes via electrostatic interactions. The hydrophobic regions of AMPs interact only weakly with the zwitterionic phospholipids in eukaryotic membranes. Moreover, cholesterol present in eukaryotic membranes protects the plasma membrane from AMP activity (modified from reference 7).*

Table I. *Half maximal inhibitory concentration (IC_{50}) of $(KLAKLAK)_2$ for various cells (24 hours of incubation).*

Cell name	Type	IC_{50} (μM)
MCF-7	Breast carcinoma	88.1
MDA-MB435S	Breast carcinoma	140.0
MDA-MB453	Breast carcinoma	191.0
SKBR3	Breast carcinoma	>320
T47D	Breast carcinoma	247.0
DU145	Prostate carcinoma	183.3
T24	Urinary bladder carcinoma	161.6
Tet21N	Neuroblastoma	167.1
Wac2	Neuroblastoma	181.3
PBL	Peripheral blood lymphocytes	>320
293	Embryonic kidney epithelial	>320

insulin (Invitrogen); T24 and DU145 cells were maintained in RPMI medium supplemented with L-glutamine (PAA Laboratories), 1% penicillin/streptomycin and 5% heat inactivated FCS. Tet21N and Wac2 cells were kindly supplied by Professor Dr. M. Schwab, Deutsches Krebsforschungszentrum, Heidelberg, Germany. Both cell lines were propagated in RPMI medium supplemented with L-glutamine, 1% penicillin/streptomycin and 5% heat inactivated FCS. 293 Human embryonic kidney epithelial cells were kindly provided by Dr. M. Kebschull, University Hospital Bonn, Germany. All other cell lines (SKBR3, MCF-7, T47D, MDA-MB453) were provided by the study group of molecular and immune therapy (AG Molekulare und Immuntherapie), University Hospital Bonn, Germany. These cell lines were cultured in DMEM supplemented with L-glutamine, 1% penicillin/streptomycin, 5% heat inactivated FCS and 0.2% insulin. Peripheral blood lymphocytes (PBL) were gained by density gradient centrifugation from whole blood and propagated in RPMI medium with 300 U/ml Interleukin-2 (IL-2; Immunotools, Friesoythe, Germany).

Determination of efficacy of targeted peptides by means of cell viability. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay utilizes the ability of metabolically active cells to convert the yellow dye MTT into insoluble purple formazan, which can be solubilized by an organic solvent. The relative viability of the cells can then be determined photometrically. Cells were seeded in 96-well plates in numbers of 1×10^4 cells/well/100 μl and incubated with peptide concentration ranging from 2.5 to 320 μM for 24 h. A negative control without peptide was prepared for each cell line (100% viability).

Four hours before the end of the incubation time, 1 μl MTT (Sigma, Steinheim, Germany) [5 mg/ml solved in phosphate buffered saline (PBS)] was added per well. At the end of the incubation

period, 50 μl MTT lysis buffer (0.1 N HCl in isopropanol) was added to non-adherent cells. For adherent cells, 80 μl of medium was removed and 50 μl of MTT lysis buffer was added. The plates were shaken for 10 to 40 min until all cells were lysed and dissolved at 600 rpm. The absorption of all samples was measured at 492 nm in an ELISA reader (Rysos anthos 2010, anthos Mikrosysteme GmbH, Krefeld, Germany).

Tumor xenograft model. The ability of $(KLAKLAK)_2$ to specifically kill tumor cells *in vivo* was tested using MDA-MB435S breast cancer-bearing nude mice. A total of 5×10^6 cells in 50 μl medium were co-injected with 250 μg peptide in 50 μl PBS into the mammary gland fat pads of 6-week-old female nude mice (Charles River, Sulzfeld, Germany). The mice were given weekly injections of 250 μg of $(KLAKLAK)_2$ in 50 μl PBS. Weekly injections of PBS alone were used in the control group. The tumors were measured with calipers every 2 to 3 days and tumor volumes were calculated.

Statistical analysis. Values are given as the mean±standard deviation. Statistical comparison between two groups of data was performed using the unpaired Student *t*-test. Statistical significance was defined as *p*≤0.05.

Results

MTT assay. The cytotoxicity of the $(KLAKLAK)_2$ peptide was determined for different breast carcinoma cell lines (MCF-7, MDA-MB435S, MDA-MB453, SKBR3, T47D), two neuroblastoma cell lines (Tet21N, Wac2), a prostate carcinoma cell line (DU145), a urinary bladder carcinoma cell line (T24), PBL and 293 human embryonic epithelial cells. Figure 2 shows the dose−response curves of all tested cell lines after incubation with the peptide for 24 h. At 320 μM, the toxicity of $(KLAKLAK)_2$ is significantly higher towards all cancer cell lines (*p*≤0.05), except for SKBR3, than towards PBL and 293 cells (Figure 3). In Table I, the cytotoxicity of $(KLAKLAK)_2$ towards the different cell lines is presented as half maximal inhibitory concentration (IC_{50}). The IC_{50} values of the control cells PBL and of the

A
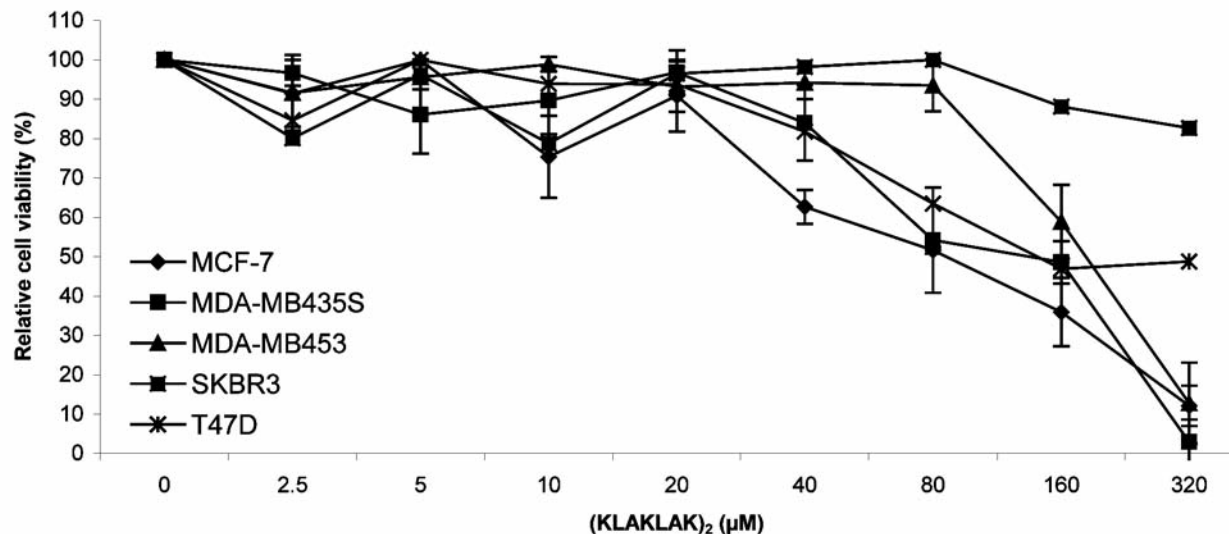
B
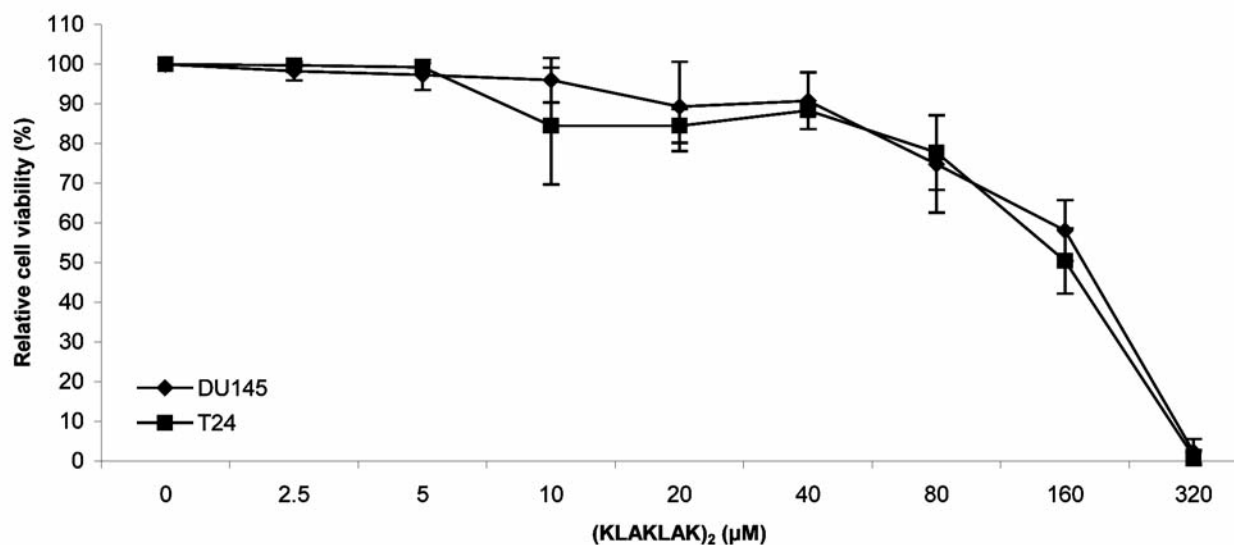
Figure 2. *Continued*

293 cells are considerably higher compared to the IC_{50} values of the tumor cells (except SKBR3) after 24 hours of incubation.

In vivo studies. The efficacy of $(KLAKLAK)_2$ was also tested *in vivo*. The results of the experiments with breast cancer-bearing mice are shown in Figure 4. Compared to the control, $(KLAKLAK)_2$ inhibits tumor growth ($p<0.001$) and prolongs overall survival ($p>0.05$) *in vivo*.

Discussion

In the study by Javadpour *et al*. (14) the 12-mer peptide $(KLAKLAK)_2$ was found to be highly cytotoxic against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, while its toxicity was remarkably lower towards human erythrocytes and 3T3 mouse fibroblasts. Here we showed that this peptide is also effective in killing human breast cancer and other tumor cells, while it
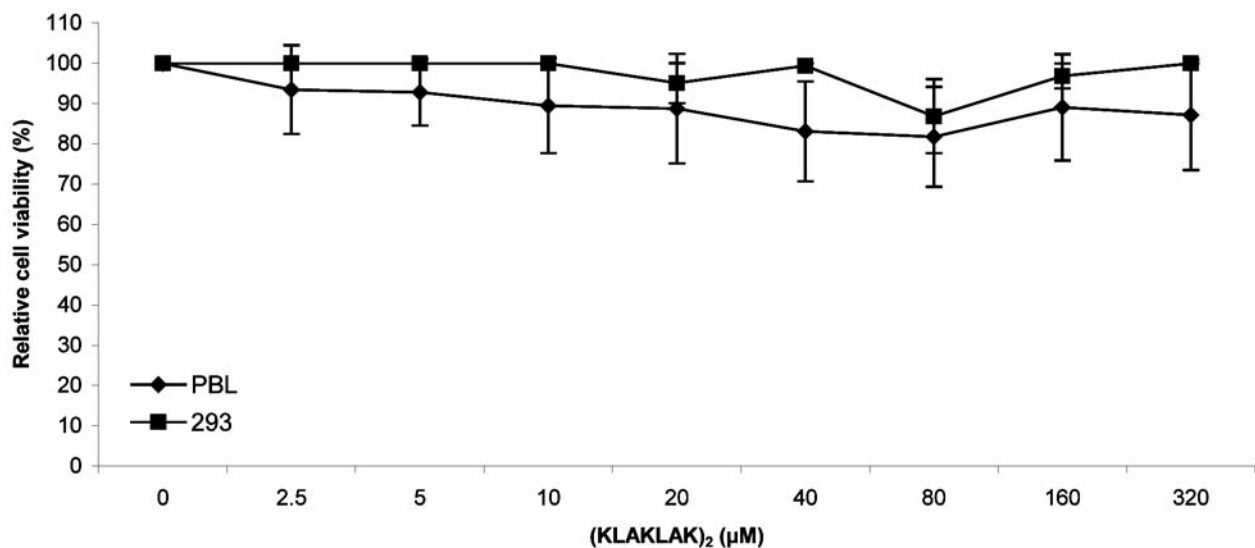
C
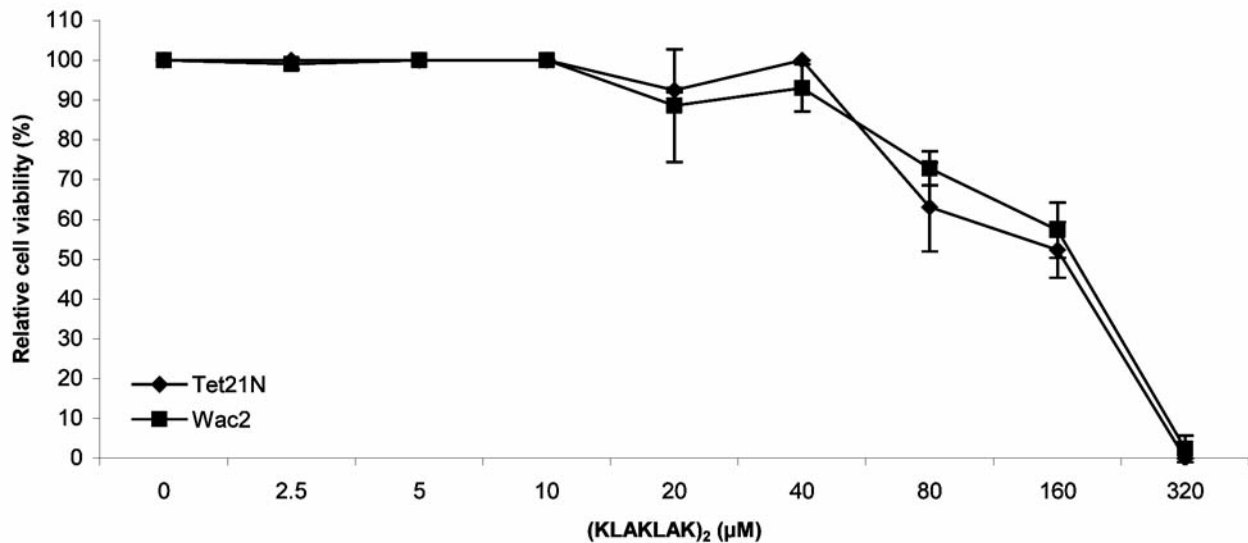
D

Figure 2. *(KLAKLAK)$_2$ inhibits growth of tumor cells at high doses while healthy cells are relatively unaffected. Cytotoxicity of (KLAKLAK)$_2$ towards breast cancer cells (A), prostate carcinoma and urinary bladder carcinoma cells (B), neuroblastoma cells (C) and non-tumor cells (D). The cells were co-incubated with (KLAKLAK)$_2$ for 24 hours. N=2-5 experiments; relative cell viability is given ±standard deviation.*

seems to be nontoxic to healthy mammalian cells (Table I, Figures 2 and 3). The best effect, *i.e.* the lowest IC$_{50}$, is seen on MCF-7 breast carcinoma cells, which seem to be most sensitive to (KLAKLAK)$_2$ activity (Table I). In contrast, the viability of the breast cancer cell line SKBR3 is relatively unaffected after 24 hours exposure to (KLAKLAK)$_2$. This

might be due to differences in the membrane composition of these cells compared to the other tumor cells which makes them less sensitive to AMP cytotoxicity.

There are several well-established mechanisms by which AMPs are known to induce cell death. Many AMPs form a transmembrane pore after accumulation on the surface or
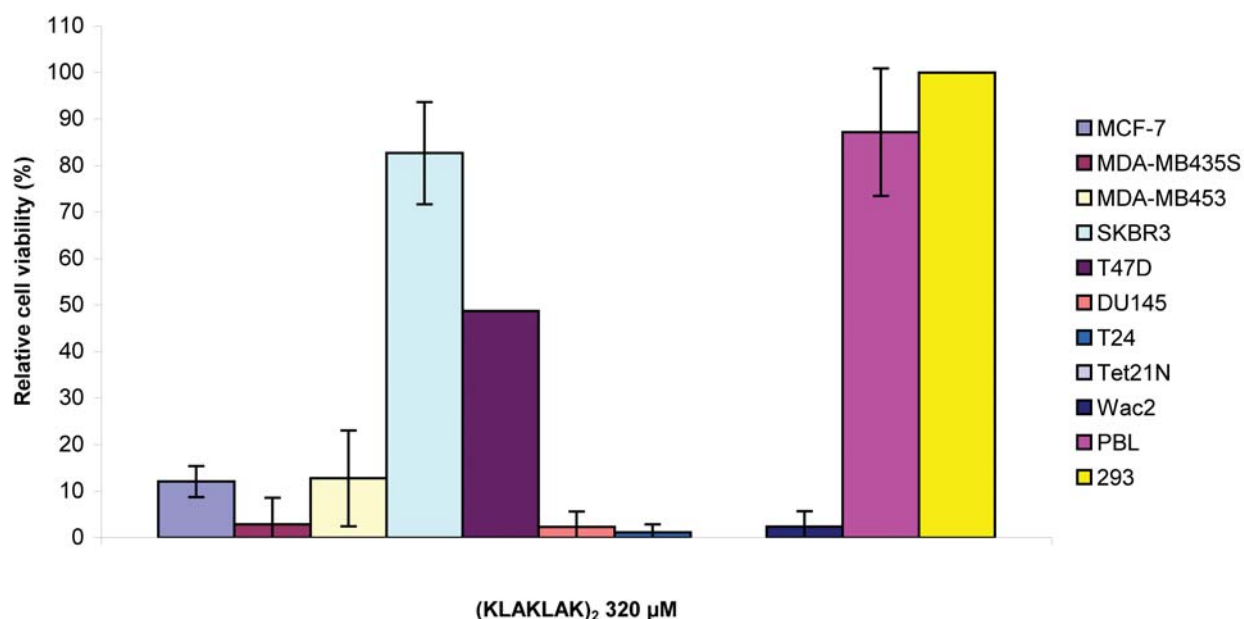
Figure 3. *High concentration of (KLAKLAK)$_2$ kills tumor cells while having little effect on healthy controls. At a concentration of 320 µM, (KLAKLAK)$_2$ reduces the viability of the tumor cell lines to 0-10%. Two exceptions are SKBR3 cells, which showed little effect, and T47D cells, of which approximately 50% remained viable. The viability of the healthy controls (PBL and 293) was barely reduced. Statistical comparison between the viability of the different cancer cell lines, PBL and 293 revealed p≤0.05 for all cancer cell lines except SKBR3.*

disrupt the membrane in a detergent-like manner (Figure 5) (15). After binding to the negatively charged membrane by electrostatic and hydrophobic interactions, the α-helical amphipathic AMPs pardaxin (9) and alamethicin (16) form a transmembrane pore via the barrel-stave mechanism, with the hydrophilic side building the channel and the hydrophobic part on the outside (Figure 5). Other α-helical amphipathic AMPs, such as magainin, have been shown to disrupt the membrane, forming a toroidal pore (17, 18) where the accumulation of the peptide on the membrane surface induces the membrane to curve inward, building a pore with peptide and phospholipids (Figure 5).

The selective action of cationic AMPs on bacteria and mammalian tumor cells compared to healthy cells is due to differences in membrane composition. As depicted in Figure 1, cationic AMPs preferentially bind to acidic lipids, such as phosphatidylglycerol and phosphatidylserines in the bacterial membrane by electrostatic interactions. In contrast, mammalian plasma membranes consist mostly of zwitterionic phospholipids, such as phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, thus carrying a net neutral charge. Compared to healthy cells, tumor cells are known to express higher levels of anionic phospholipids, such as phosphatidlyserines (19, 20) and *O*-glycosylated mucins (21). The resulting overall negative charge renders tumor cells more sensitive towards cationic AMPs. Moreover, the

negative membrane potential of tumor cells makes them more sensitive to disruption by AMPs (22). Other factors may also include increased membrane fluidity (23) and a greater number of microvilli (24), resulting in a greater surface area available for interaction with AMPs.

The cytotoxicity of AMPs towards cancer cells may be caused by the aforementioned effects on the plasma membrane or by distortion of the mitochondrial membrane after uptake into the cytoplasm by the same mechanisms. The disruption and depolarization of the plasma membrane leads to leakage of ions and metabolites out of the cell and finally to necrosis. In a study by Papo *et al*. (25), the cationic amphipathic peptide D-K$_6$L$_6$ specifically induced necrotic death in tumor cells while sparing healthy cells *in vivo*. However, for example for magainins, which are also cationic amphipathic AMPs, it is not clear whether they kill tumor cells primarily through plasma membrane lysis, induction of apoptosis, or both (3). The AMPs BMAP-27 and -28 were shown to induce apoptosis after disruption of membrane integrity (26). In a study by Horton and Kelley (27), the D-(KLAKLAK)$_2$ peptide was shown to exhibit mitochondrial localization in HeLa cells. The mitochondrial membrane is highly susceptible to disruption by (KLAKLAK)$_2$ as it has a high anionic phospholipid content and a large negative membrane potential (28, 29). The consequential loss of mitochondrial membrane integrity
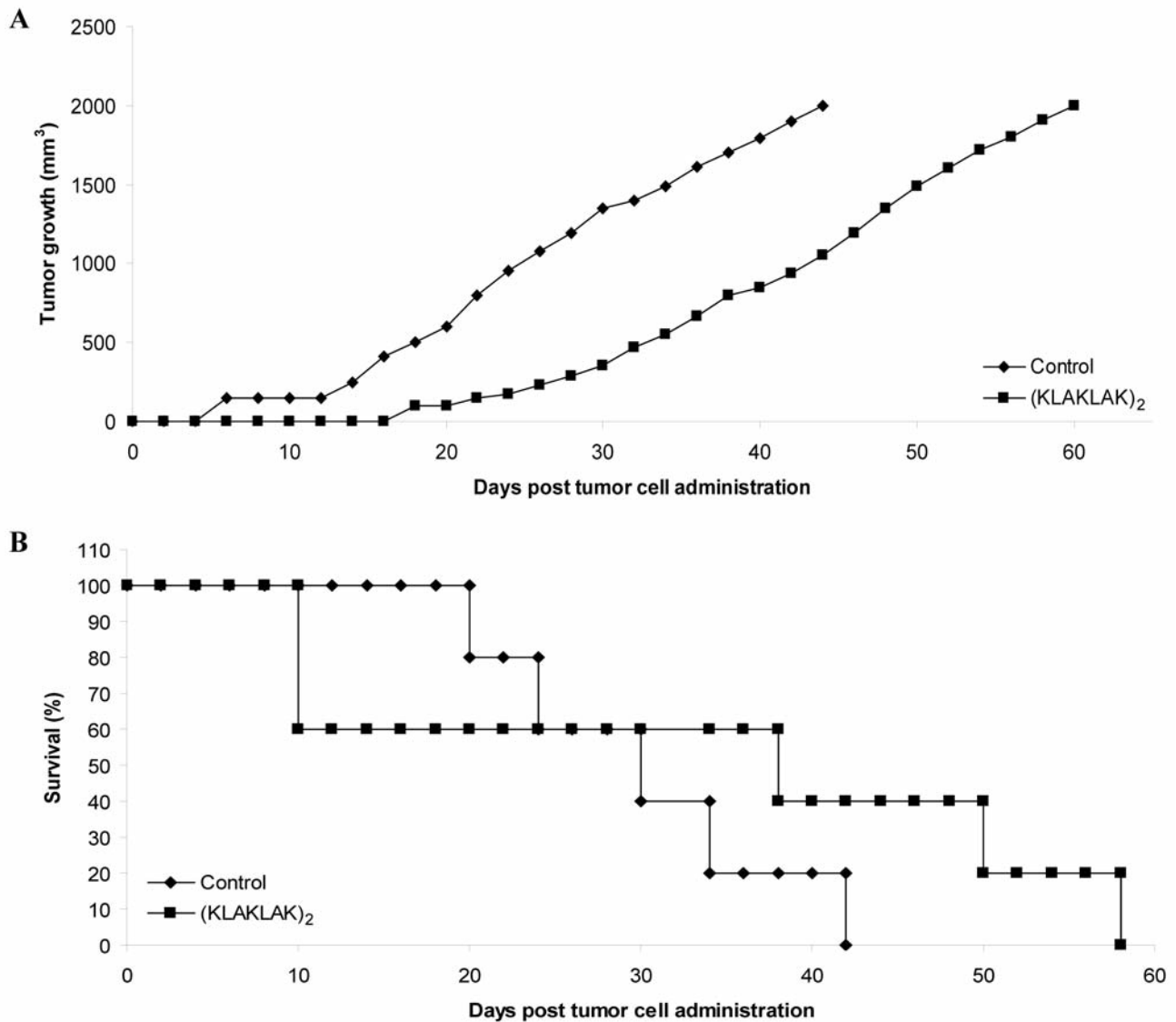
Figure 4. *(KLAKLAK)$_2$ inhibits tumor growth and prolongs survival in vivo. Nude mice (n=6) were injected with MDA-MB435S tumor cells into the mammary gland fat pads. A: Tumor growth in mice which received weekly injections of PBS (control) or the peptide (KLAKLAK)$_2$. B: Overall survival of the tumor-bearing mice which received weekly injections of PBS (control) or the peptide (KLAKLAK)$_2$.*
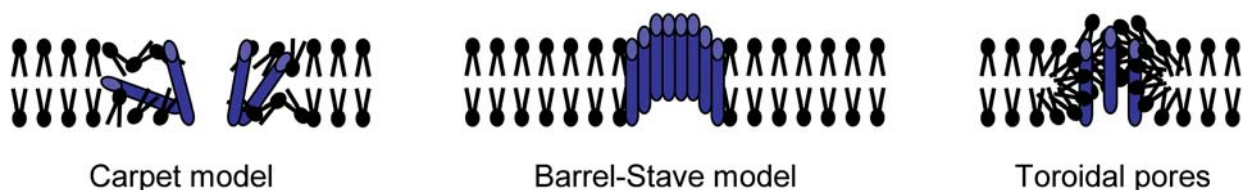

Figure 5. *Mechanism of action of antimicrobial peptides (AMPs). In the carpet model, the cationic AMPs first bind to the membrane surface via electrostatic interactions and cover it in a carpet-like manner. After a critical threshold concentration is reached, the AMPs insert into the membrane and distort it, finally leading to micellization. In the barrel-stave model, the hydrophobic part of the AMP interacts with the membrane resulting in the formation of a transmembrane pore with the hydrophilic part of the AMP facing the inner channel. In the third model, the toroidal pore model, the membrane is caused to curve inward, resulting in the formation of a pore made up of AMPs and lipid head groups (modified from references 3 and 15).*

results in the leakage of all mitochondrial contents into the cytoplasm. The release of cytochrome c and apoptosis-inducing factor-1 leads to the activation of downstream caspases, inducing apoptosis (30).

In order to test the efficacy of (KLAKLAK)$_2$ *in vivo*, nude mice bearing MDA-MB435S breast cancer tumors were treated with (KLAKLAK)$_2$ or PBS as a control for 60 days (Figure 4). Treatment with (KLAKLAK)$_2$ was able to delay tumor growth compared to treatment with PBS (Figure 4 A). Moreover, mice treated with the peptide survived 16 days longer in comparison to the control mice (Figure 4 B).

Further research on the action of the (KLAKLAK)$_2$ peptide and its potential use in therapy for breast and other types of cancer is of great value. In order to increase its specificity and effectivity, (KLAKLAK)$_2$ can be coupled to cell–penetrating peptides or peptide sequences directed against tumor cells to specifically induce apoptosis in target cells as shown in several *in vitro* and *in vivo* studies (10, 13, 31-34). Moreover, using AMPs in tumor therapy in combination with conventional drugs might be useful. This might reduce the required dose of *e.g.* chemotherapeutic drugs and thus their associated side-effects. Another possibility would be to use AMPs in gene therapy, transferring AMP-encoding genes into cancer cells (3).

Acknowledgements

References

1 American Cancer Society Cancer Facts & Figures 2010. Atlanta: American Cancer Society, 2010.

2 American Cancer Society Breast Cancer Facts & Figures 2009-2010. Atlanta: American Cancer Society, 2009.

3 Hoskin DW and Ramamoorthy A: Studies on anticancer activities of antimicrobial peptides. Biochim Biophys Acta *1778*: 357-375, 2008.

4 Pálffy R, Gardlík R, Behuliak M, Kadasi L, Turna J and Celec P: On the physiology and pathophysiology of antimicrobial peptides. Mol Med *15*: 51-59, 2009.

5 Epand RM and Vogel HJ: Diversity of antimicrobial peptides and their mechanisms of action. Biochim Biophys Acta *1462*: 11-28, 1999.

6 Zasloff M: Antimicrobial peptides of multicellular organisms. Nature *415*: 389-395, 2002.

7 Matsuzaki K: Why and how are peptide–lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. Biochim Biophys Acta *1462*: 1-10, 1999.

8 Tytler EM, Anantharamaiah GM, Walker DE, Mishra VK, Palgunachari MN and Segrest JP: Molecular basis for prokaryotic specificity of magainin-induced lysis. Biochemistry *34*: 4393-4401, 1995.

9 Hallock KJ, Lee DK, Omnaas J, Mosberg HI and Ramamoorthy A: Membrane composition determines pardaxin's mechanism of lipid bilayer disruption. Biophys J *83*: 1004-1013, 2002.

10 Ellerby HM, Arap W, Ellerby LM, Kain R, Andrusiak R, Rio GD, Krajewski S, Lombardo CR, Rao R, Ruoslahti E, Bredesen DE and Pasqualini R: Anticancer activity of targeted pro-apoptotic peptides. Nat Med *5*: 1032-1038, 1999.

11 Mai JC, Mi Z, Kim SH, Ng B and Robbins PD: A proapoptotic peptide for the treatment of solid tumors. Cancer Res *61*: 7709-7712, 2001.

12 Law B, Quinti L, Choi Y, Weissleder R and Tung CH: A mitochondrial targeted fusion peptide exhibits remarkable cytotoxicity. Mol Cancer Ther *5*: 1944-1949, 2006.

13 Kim HY, Kim S, Youn H, Chung JK, Shin DH and Lee K: The cell penetrating ability of the proapoptotic peptide, KLAKLAKKLAKLAK fused to the *N*-terminal protein transduction domain of translationally controlled tumor protein, MIIYRDLISH. Biomaterials *32*: 5262-5268, 2011.

14 Javadpour MM, Juban MM, Lo WC, Bishop SM, Alberty JB, Cowell SM, Becker CL and McLaughlin ML: *De novo* antimicrobial peptides with low mammalian cell toxicity. J Med Chem *39*: 3107-3113, 1996.

15 Papo N and Shai Y: Host defense peptides as new weapons in cancer treatment. Cell Mol Life Sci *62*: 784-790, 2005.

16 Stella L, Burattini M, Mazzuca C, Palleschi A, Venanzi M, Coin I, Peggion C, Toniolo C and Pispisa B: Alamethicin interaction with lipid membranes: a spectroscopic study on synthetic analogues. Chem Biodivers *4*: 1299-1312, 2007.

17 Ludtke SJ, He K, Heller WT, Harroun TA, Yang L and Huang HW: Membrane pores induced by magainin. Biochemistry *35*: 13723-13728, 1996.

18 Nguyen KT, Le Clair SV, Ye S and Chen Z: Molecular interactions between magainin 2 and model membranes *in situ*. J Phys Chem B *113*: 12358-12363, 2009.

19 Utsugi T, Schroit AJ, Connor J, Bucana CD and Fidler IJ: Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. Cancer Res *51*: 3062-3066, 1991.

20 Dobrzyńska I, Szachowicz-Petelska B, Sulkowski S and Figaszewski Z: Changes in electric charge and phospholipids composition in human colorectal cancer cells. Mol Cell Biochem *276*: 113-119, 2005.

21 Ho SB, Niehans GA, Lyftogt C, Yan PS, Cherwitz DL, Gum ET, Dahiya R and Kim YS: Heterogeneity of mucin gene expression in normal and neoplastic tissues. Cancer Res *53*: 641-651, 1993.

22 Cruciani RA, Barker JL, Zasloff M, Chen HC and Colamonici O: Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation. Proc Natl Acad Sci USA *88*: 3792-3796, 1991.

23 Sok M, Sentjurc M and Schara M: Membrane fluidity characteristics of human lung cancer. Cancer Lett *139*: 215-220, 1999.

24 Chaudhary J and Munshi M: Scanning electron microscopic analysis of breast aspirates. Cytopathology *6*: 162-167, 1995.

25 Papo N, Seger D, Makovitzki A, Kalchenko V, Eshhar Z, Degani H and Shai Y: Inhibition of tumor growth and elimination of multiple metastases in human prostate and breast xenografts by systemic inoculation of a host defense-like lytic peptide. Cancer Res *66*: 5371-5378, 2006.

26 Risso A, Zanetti M and Gennaro R: Cytotoxicity and apoptosis mediated by two peptides of innate immunity. Cell Immunol *189*: 107-115, 1998.

27 Horton KL and Kelley SO: Engineered apoptosis-inducing peptides with enhanced mitochondrial localization and potency. J Med Chem *52*: 3293-3299, 2009.

28 Daum G: Lipids of mitochondria. Biochim Biophys Acta *822*: 1-42, 1985.

29 de Kroon AI, Dolis D, Mayer A, Lill R and de Kruijff B: Phospholipid composition of highly purified mitochondrial outer membranes of rat liver and Neurospora crassa. Is cardiolipin present in the mitochondrial outer membrane? Biochim Biophys Acta *1325*: 108-116, 1997.

30 Bhutia SK and Maiti TK: Targeting tumors with peptides from natural sources. Trends Biotechnol *26*: 210-217, 2008.

31 Marks AJ, Cooper MS, Anderson RJ, Orchard KH, Hale G, North JM, Ganeshaguru K, Steele AJ, Mehta AB, Lowdell MW and Wickremasinghe RG: Selective apoptotic killing of malignant hemopoietic cells by antibody−targeted delivery of an amphipathic peptide. Cancer Res *65*: 2373-2377, 2005.

32 Watkins CL, Brennan P, Fegan C, Takayama K, Nakase I, Futaki S and Jones AT: Cellular uptake, distribution and cytotoxicity of the hydrophobic cell penetrating peptide sequence PFVYLI linked to the proapoptotic domain peptide PAD. J Control Release *140*: 237-244, 2009.

33 Dufort S, Sancey L, Hurbin A, Foillard S, Boturyn D, Dumy P and Coll JL: Targeted delivery of a proapoptotic peptide to tumors *in vivo*. J Drug Target *19*: 582-588, 2011.

34 Lemeshko VV: Potential−dependent membrane permeabilization and mitochondrial aggregation caused by anticancer polyarginine−KLA peptides. Arch Biochem Biophys *493*: 213-220, 2010.